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INTERACTION OF BENZODIAZEPINE DERIVATIVES WITH BOVINE SERUM ALBUMIN—II.

CIRCULAR DICHROISM STUDIES*†

WALTER E. MÜLLER and UWE WOLLERT

Pharmakologisches Institut der Universität Mainz, D 65 Mainz, Obere Zahlbacher Str. 67, G.F.E.

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Abstract—The interaction of benzodiazepine derivatives with bovine serum albumin (BSA) was studied by circular dichroism (CD) measurements. Most of the investigated benzoidiazepines show biphasic extrinsic Cotton effects, which are largely influenced by raising pH from 6·60 to 8·20. Quantitative estimation of the CD data pointed out that there are several binding sites on the BSA molecule. The CD data do not differ very much from those found for the interaction of the drugs with human serum albumin (HSA). Therefore it is suggested that at least a part of the benzodiazepine binding sites on the BSA molecule has similar properties to the single binding site on the HSA molecule. The extrinsic Cotton effects of the benzodiazepines in the presence of BSA are influenced by fatty acids and sodium dodecyl sulfate in a similar way as by the pH-value of the solution. This is explained by a similar influence of the substances and of pH on the protein conformation.

In recent years it has been demonstrated that circular dichroism (CD) measurements are an excellent tool for studying drug–protein interactions [2, 3]. This method depends on the phenomenon that the binding of a small symmetric ligand to an asymmetric macromolecule, e.g. a protein, can induce optical activity in the ligand molecule, originating by a perturbation of electronic transitions of the ligand by an asymmetric locus of the macromolecule [2–4].

By CD measurements differences in the interaction of organic molecules with human and bovine serum albumin (HSA and BSA) could be demonstrated [5–8], especially for substances whose affinities and numbers of binding sites for both albumins do not differ very much, as found by other methods [5, 6].

In previous papers [9, 10] it has been shown that human and bovine serum albumin differ in the binding behaviour for benzodiazepines in an unusually high degree, e.g. there are differences in the affinities [9, 10], in the numbers of binding sites [9, 10], and in substituent influences [9]. We have suggested that the main reason for these large differences is that the specific binding site, found on the HSA molecule [11, 12], does not exist on the BSA molecule [9]. Nearly all investigated benzodiazepine derivatives have two or three equivalent binding sites on the BSA molecule [9].

The interaction of the benzodiazepine derivatives with HSA produces typical biphasic circular dichroism spectra [12, 13], extrinsic in origin [14]. We think therefore that CD measurements are a very suitable method to get more information on the binding of the benzodiazepines to BSA and on the large differences of both albumins in the binding behaviour of these substances.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) was obtained from Behringwerke, Marburg (quality: "trocken, reinst"), electrophoretic purity 100%. Medazepam was obtained from Hoffmann–La Roche AG, Grenzach, and prazepam from Gödecke, Freiburg. All other benzodiazepines were obtained from the manufacturers as described in the first paper [9]. For the chemical formulas of the derivatives see Ref. 13. All other chemicals were of reagent grade. All solutions were made up with distilled water.

Circular dichroism measurements. Circular dichroism (CD) measurements were made at 27° with a Cary 61 spectropolarimeter, which was calibrated with D-10-camphorsulfonic acid. All spectra were recorded in cylindrical cells with 10-mm pathlength, using a full-scale deflection of 0·02° or 0·05° θ and a spectral bandwidth of 2 nm. Results are expressed as molar ellipticities $[\theta]$ (degr. $\times$ cm^2 $\times$ dmole^{-1}), calculated with reference to the BSA concentration, using a molecular weight of 69,000. The solutions for the CD measurements were prepared as described by Müller and Wollert [13]. The final BSA concentration was always $1{\cdot}31 \times 10^{-5}$ M (0·09%) and the drug concentrations were varied. All solutions were made with 1/15 M phosphate buffer, pH ranging from 6·60 to 8·20, and adjusted to the desired pH-value with 1 M HCl or 1 M NaOH. Each CD spectrum reported is the average of three observations.

Binding data from CD measurements. The CD data were analysed according to the method of Rosen [15], using a plot of the molar ellipticity versus the drug concentration (Fig. 5). The binding was characterized by the number of binding sites, n, and the binding constant K (obtained from the total binding constant

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K_1 by multiplying with the BSA concentration) [12, 16]. K_1 and n were determined by the reciprocal plot [7] as described in the preceding paper [9].

Calculations. The molar ellipticities were obtained from the equation [7]:

$$[\theta] = \frac{100 \times \theta}{l \times C}$$

where θ = the observed ellipticity using the CD spectra of BSA alone as references, l = the pathlength in cm, and C = the molar concentration.

The anisotropy or dissymmetry factors (g-values) [18] were calculated from the relationship [7]:

$$g = \frac{[\theta]_\lambda}{3300 \times \varepsilon_\lambda}$$

where $[\theta]_\lambda$ = the molar ellipticity at the wavelength of the CD maximum, calculated with respect to the total concentrations of the benzodiazepines, and ϵ_λ = the molar extinction coefficient of the benzodiazepines at the wavelength of the CD maximum in 1/15 M phosphate buffer [13]. The g-values (Table 1) are not corrected for the concentration of the drug bound as done by Chignell [7] and are not corrected for binding induced alterations of the absorbance of the drugs.

RESULTS

In nearly all cases the complex formation between most of the investigated benzodiazepines and BSA produced biphasic extrinsic Cotton effects (Figs. 1, 2 and Table 1) with two bands whose ellipticities were more positive or more negative than those of the CD spectrum of BSA alone. The intensities differ very much (see the g-values in Table 1). Although these g-values are uncorrected, they give better information than the molar ellipticities (table 1) alone about the degree of the optical perturbation of the electronic transitions of the benzodiazepines, because the molar

Table 1. CD-data of the benzodiazepine BSA complex at pH 7·40

Substance (5×10^{-5} M)	λ_{max} CD (nm)	$[\theta] \times 10^{-3}$ $\bar{x} \pm SX$ ($n = 3$)	$g \times 10^4$
Oxazepam	258	+57·50 ± 1·21	+3·136
	317	−23·35 ± 0·18	−6·715
Lorazepam	265	+55·09 ± 1·39	+8·543
	320	−16·71 ± 1·33	−8·261
Chlordiazepoxid	258	+44·47 ± 2·59	+1·005
	320	−1·63 ± 0·42	−0·280
Medazepam	268	+32·40 ± 1·75	+3·882
Prazepam	258	−31·20 ± 1·63	−1·927
	312	+10·26 ± 0·60	+4·160
Clonazepam	258	−21·90 ± 0·42	−1·224
	335	+10·08 ± 0·18	+1·226
Diazepam	258	+17·68 ± 1·45	+0·958
	317	−4·80 ± 2·40	−1·558
Nitrazepam	258	−10·86 ± 1·39	−0·455
	305	+9·47 ± 0·54	+0·645
Bromazepam	258	+9·65 ± 0·60	+0·581
Dipotassium clorazepate	258	+5·01 ± 1·21	+0·274
	268	−7·66 ± 1·09	−0·774
	303	−12·49 ± 1·57	−4·819
Demoxepam	258	+3·44 ± 1·21	+0·154
Tetrazepam	258	−3·62 ± 1·27	−0·224

Molar ellipticities ($[\theta]$), calculated in respect to the BSA concentration (0·09%), and anisotropy factors (g-values), calculated in respect to the drug concentration (5×10^{-5} M), at the wavelengths of the CD-maxima (λ_{max} CD).

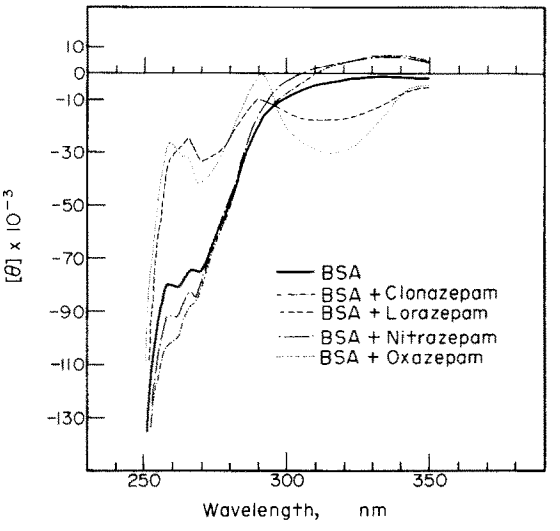

Fig. 1. CD spectra of clonazepam, lorazepam, nitrazepam, and oxazepam (5×10^{-5} M) in presence of BSA (0·09%) at pH 7·40. Ordinate: molar ellipticity calculated with reference to the BSA concentration. Abscissa: wavelength in nm.

ellipticity is directly proportional to the extinction coefficients of the substances [13].

The signs of the biphasic extrinsic Cotton effects at pH 7·40 are not identical for all benzodiazepines. Oxazepam, lorazepam, chlordiazepoxid, and diazepam have a positive CD band at shorter wavelengths and a negative at longer wavelengths, whereas the signs of these CD bands are inversed in the case of prazepam, clonazepam, and nitrazepam (Figs. 1, 2 and Table 1). Dipotassium clorazepate has a third CD band near 270 nm (Table 1), which is similar to that of demoxepam in the presence of HSA [13]. The origin of both bands is not yet clear [13].

The induced Cotton effects of the benzodiazepines bound to BSA are intensively influenced by fatty acids and sodium dodecyl sulfate (Table 2). Whereas the wavelength positions of the bands remain unchanged,

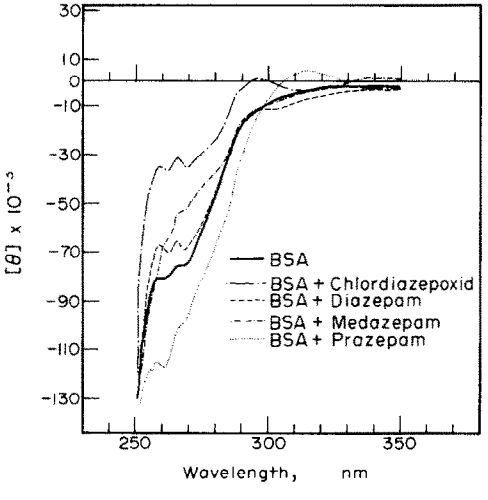

Fig. 2. CD spectra of chlordiazepoxid, diazepam, medazepam, and prazepam (5×10^{-5} M) in presence of BSA (0·09%) at pH 7·40. Ordinate and abscissa: see Fig. 1.

Table 2. Influences of fatty acids and sodium dodecyl sulfate on the extrinsic cotton effects of benzodiazepines bound to BSA.

Substance [5 × 10^{-5} M]	λ_{max} CD [nm]	$[\theta] \times 10^{-3}$ $x \pm sx$ ($n = 3$) Control	Oleic acid [10^{-4} M]	Caprylic acid [10^{-4} M]	Stearic acid [10^{-4} M]	Na^+-Dodecyl sulfate [10^{-4} M]
Oxazepam	258	+57·50 ± 1·21	+14·06 ± 3·14	+50·26 ± 4·77	+32·76 ± 3·32	+15·09 ± 0·36
	317	−23·35 ± 0·18	+1·03 ± 1·03	−32·76 ± 1·57	−11·89 ± 0·91	+1·99 ± 0·54
Lorazepam	265	+55·09 ± 1·39	−22·14 ± 3·14	+63·36 ± 2·11	−10·08 ± 1·21	−7·06 ± 0·54
	320	−16·71 ± 1·33	+6·22 ± 0·91	−19·67 ± 0·72	+5·25 ± 1·81	+9·23 ± 0·18
Medazepam	268	+32·40 ± 1·75	−8·27 ± 1·03	−6·03 ± 0·36	+12·49 ± 1·09	−24·14 ± 2·11
Prazepam	258	−31·20 ± 1·63	−30·17 ± 1·75	−45·26 ± 1·93	−50·81 ± 3·62	−52·80 ± 1·51
	312	+10·26 ± 0·60	+6·52 ± 0·48	+12·67 ± 1·51	+8·03 ± 2·78	+13·58 ± 0·0
Diazepam	258	+17·68 ± 1·45	−8·87 ± 1·69	−0·60 ± 2·47	−10·08 ± 1·03	−17·70 ± 2·29
	317	−4·80 ± 2·40	+3·02 ± 0·60	−1·81 ± 2·11	+4·22 ± 0·72	+10·68 ± 1·45
Tetrazepam	258	−3·62 ± 1·27	−56·30 ± 11·83	−24·14 ± 1·27	−77·72 ± 0·72	−81·46 ± 7·54

The molar ellipticities ($[\theta]$), calculated in respect to the BSA concentration (0·09%) of some benzodiazepines (5 × 10^{-5} M) bound to BSA at pH 7·40 in presence of oleic acid, caprylic acid, stearic acid and sodium dodecyl sulfate (10^{-4} M) at the wavelengths of the CD-maxima (λ_{max} CD).

the intensities and the signs of the extrinsic Cotton effects were altered (Table 2) in a qualitatively similar way. The signs of the CD bands of the N_1-substituted benzodiazepines (medazepam, prazepam, diazepam, and tetrazepam) are influenced so that negative and positive CD bands appear at shorter and longer wavelengths respectively (Table 2). The CD spectra of the two N_1-not substituted benzodiazepines (oxazepam and lorazepam) are mainly influenced by oleic acid, stearic acid, and dodecyl sulfate in a qualitatively similar but quantitatively smaller extent (table 2). Furthermore, stearic acid and sodium dodecyl sulfate have the greatest influence on the CD spectra of the N_1-substituted benzodiazepines (Table 2). In the region between 350 and 250 nm, the fatty acids and sodium dodecyl sulfate have no or negligible influence on the CD spectrum of BSA alone.

The CD spectra of the benzodiazepines are also influenced by the pH of the solutions (Figs. 3 and 4). The signs of the induced Cotton effects of nearly all benzodiazepines are changed by raising pH from 6·60 to 8·20 (Figs. 3 and 4). The extrinsic Cotton effects of oxazepam and dipotassium clorazepate are slightly increased by raising the pH-value (Fig. 4), whereas the ellipticity of lorazepam is only slightly decreased at higher pH-values (Fig. 4). The ultraviolet spectra of the benzodiazepines [12] and the CD spectrum of BSA alone are not or negligibly changed between pH 6·60 and 8·20.

The extrinsic Cotton effects of the benzodiazepines in the presence of BSA depend on the benzodiazepine concentration (Fig. 5), as shown for the three derivatives, which exhibit the highest perturbation of the short wavelength CD band (Table 1). Whereas the curve of the ellipticity of medazepam versus the total concentration of the drug seems to have a saturation point at 6 × 10^{-5} M (corresponding a molar drug/protein ratio of 4·6), for lorazepam and oxazepam no saturation is obtained up to 10^{-4} M (corresponding a molar drug/protein ratio of 7·6) (Fig. 5). The concentration dependence of the extrinsic Cotton effects

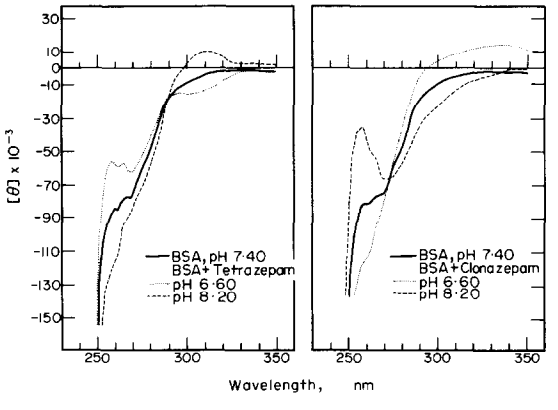

Fig. 3. pH dependence of the CD spectra of two benzodiazepines in presence of BSA (0·9%). Left side: tetrazepam (5 × 10^{-5} M). Right side: clonazepam (5 × 10^{-5} M). Ordinate: molar ellipticity calculated with reference to the BSA concentration. Abscissa: wavelength in nm.

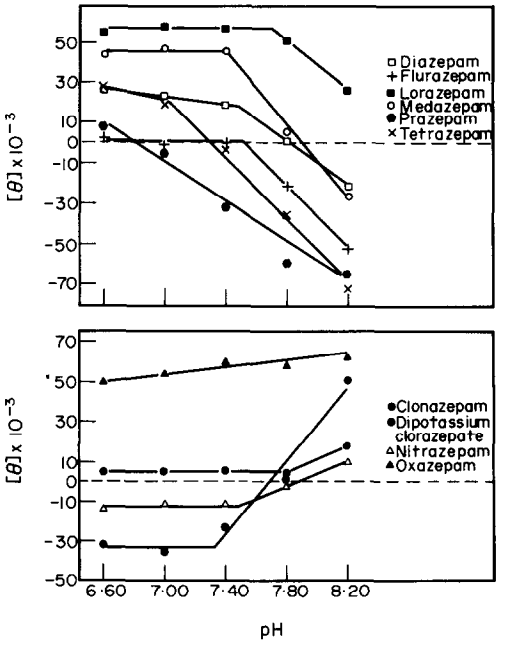

Fig. 4. pH dependence of the intensities of the short wavelength CD bands near 260 nm (Table 1) of the benzodiazepines (5 × 10^{-5} M) in presence of BSA (0·09%). Ordinate: molar ellipticity calculated with reference to the BSA concentration. Abscissa: pH.

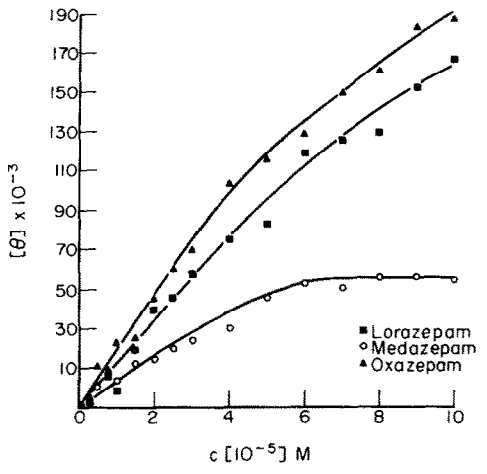

Fig. 5. The dependence of the short wavelength CD bands near 260 nm (Table 1) on the benzodiazepine concentration at pH 7·40. Ordinate: molar ellipticity calculated with respect to the BSA concentration (0·09%). Abscissa: total concentration (c) of the benzodiazepines.

of these three substances (Fig. 5) was used to determine the number of binding sites n and the binding constant K, as described in Methods. All other benzodiazepine derivatives could not be evaluated by this method, because of their small extrinsic Cotton effects at pH 7·40 (Table 1). The number of binding sites n and the binding constants K obtained from CD measurements at pH 7·40 with BSA 0·09% are not identical to those obtained from gel filtration measurements at pH 7·40 with BSA 1% [9] as shown in Table 3.

DISCUSSION

Following the method of Rosen [15], CD measurements can be used to determine quantitative binding data, e.g. binding constants and the number of binding sites (see Methods). The binding constants and the number of binding sites found by CD measurements in this study, are not identical to those found by gel filtration measurements (Table 3). This is not surprising, because the BSA concentration of the CD measurements is more than 10-fold smaller than the BSA concentration of the gel filtration measurements [9]. It is known that by increasing albumin concentrations the affinity and the number of binding sites of a ligand can decrease [19]. This can be explained by mutual interactions of the albumin side chains [19]. At lower protein concentrations the mutual interactions are diminished, the affinity can increase and new binding sites will become available [19]. This could be the case for the interaction of the benzodiazepines with BSA, especially since this interaction is relatively unspecific [9]. Furthermore the results obtained by the method of Rosen [15] depend on a tangent of the curve, describing the dependence of the extrinsic Cotton effects on the ligand concentratio [15, 12]. Because this tangent must be drawn by hand, the precision of the method is limited. This may explain the differences of the results obtained by gel filtration and CD measurements (Table 3), too.

Table 3. Comparison of binding constants obtained from gel filtration or circular dichroism measurements*

	Gel filtration			CD measurements		
Substance	n	K	K/n	n	K	K/n
Lorazepam	2·9	5·95	2·05	5·4	47·46	8·82
Medazepam				4·3	251·92	59·00
Oxazepam	2·0	5·33	2·64	5·5	53·47	9·74

* The number of binding sites, n, the binding constants K(obtained from the total binding constant K_1 by multiplying with the BSA concentration), and the binding constant K corrected for the number of binding sites n (K/n-value), obtained from gel filtration or circular dichroism measurements at pH 7·40. The values of the gel filtration measurements were taken from Ref. 9. The values of the CD measurements were obtained from reciprocal plots, as described in methods. The equations of the regression lines in the reciprocal plot are for:

Lorazepam
$1/\bar{r} = 0{\cdot}186 + 0{\cdot}276\ 1/c_f$; $r = 0{\cdot}7762$
Medazepam
$1/\bar{r} = 0{\cdot}233 + 0{\cdot}052\ 1/c_f$; $r = 0{\cdot}8867$
Oxazepam
$1/\bar{r} = 0{\cdot}182 + 0{\cdot}245\ 1/c_f$; $r = 0{\cdot}8805$
r = correlation coefficient

On the other hand, in the case of the interaction of the benzodiazepines with HSA, using the same protein concentration relation, we have found comparable binding data with both methods for most of the investigated benzodiazepines [12]. In regard to the discussion above, this is very remarkable and supports our suggestion of a very specific interaction of the benzodiazepines with the HSA molecule [11–14]. This shows in summary that care has to be taken to draw conclusions from drug binding studies done with different albumin concentrations.

CD measurements are a very sensitive method to study drug protein interactions, because small changes of the drug/protein complex can have large influences on the CD spectra [6, 7, 12]. It has been shown that the benzodiazepines differ in their binding behaviour to BSA and HSA respectively [9], therefore one has to expect large differences of the CD spectra of the benzodiazepine derivatives in presence of both albumins. But surprisingly in the case of some benzodiazepines there is only a small difference between the CD spectra in the presence of BSA or HSA (see the CD spectra of oxazepam and lorazepam in Fig. 1 and the g-values in Table 1. The corresponding data for the interaction with HSA can be found in Ref. 13). On the other hand, the induced CD spectra of the N_1-substituted derivatives and of chlordiazepoxid at pH 7·40 (Fig. 2, Table 1) differ quantitatively as well as qualitatively from those found in the presence of HSA [13]. But regarding the extrinsic Cotton effects at pH 6·60 (Fig. 4) only quantitative differences can be seen, because all benzodiazepines, except the two nitroderivatives, nitrazepam and clonazepam, have positive short wavelength CD bands, similar to the CD spectra of the drugs in the presence of HSA at pH 6·60 [12]. The lower intensities of the induced Cotton effects can be explained by the lower affinities of most of the drugs to BSA [9]. For example, bromazepam and flurazepam, which are only weakly bound at pH 7·40 [9], show no or only small extrinsic Cotton effects (Table 1). At pH 8·20 the largely increased binding of flurazepam [9] is accompanied by a high increase of the extrinsic Cotton effects (Fig. 4). The

influence of pH on the CD spectra of the benzodiazepines in the presence of HSA was explained by the low alcaline structure transition of the HSA molecule [12], called N → B transition [20]. Obviously, the N → B transition of the BSA molecule [21] has a similar influence on the CD spectra of the benzodiazepines bound to BSA. But the influence of pH on the extrinsic Cotton effects is larger and begins at lower pH-values in the experiments with BSA (Fig. 4). The differences of the CD spectra of the benzodiazepines in the presence of both albumins at pH 7·40 seem to depend more on the N → B transition of the BSA molecule than on another kind of perturbation of the electronic transitions of the benzodiazepine molecule. The CD spectra of most of the benzodiazepines in presence of BSA lead to the suggestion that the benzodiazepine binding site or sites on the BSA molecule and the chemical environment of this binding sites resemble the binding site on the HSA molecule [11, 12] more than they differ from this binding site as suggested previously [9].

The CD spectra in the presence of BSA, resembling in part qualitatively those in the presence of HSA [13], result in several binding sites (Table 3). This is in large contrast to the results with HSA, where only one very specific binding site was found [12]. The CD spectra in the presence of HSA are very sensitive to change in the structure of the drugs as well as in the protein conformation [12, 13]. It seems rather unlikely that there are about five such specific binding sites on the BSA molecule (Table 3). The assumption that not all five binding sites found by the method of Rosen [15] reflect really optical active binding sites is supported by the following considerations. The basic assumption of the method of Rosen [15] is that the increase of the extrinsic Cotton effects is directly proportional to the concentration of drug bound. This means that in a set of several equivalent binding sites, with identical association constants, all binding sites must have a similar contribution to the Cotton effects, if they should be accurately detectable by the method of Rosen [15]. At present we can not exclude that this is not the case. Furthermore it seems possible that only a few of the five binding sites found by the method of Rosen [15] have a contribution to the observed Cotton effects at all. This can be the case because in a set of several equivalent binding sites, where only a few have a contribution to the Cotton effects, all binding sites will be determined by this method.

To specify these considerations we have measured the extrinsic Cotton effects of some benzodiazepines in the presence of several fatty acids and sodium dodecyl sulfate. All these substances are known to be bound strongly to BSA to a very high degree at several binding sites [22, 23], whose numbers differ for the various substances [22]. The kind of interaction of these substances with the albumin binding of other ligands could be competition phenomenon [24–27] as well as displacement or increase of the binding, originated by conformation changes of the albumin molecule, induced by the binding of the substances [24, 26].

The influence of these substances on the CD spectra of the benzodiazepines in the presence of BSA is qualitatively similar (Table 2). There seems to be no evidence for a competition between these substances and the benzodiazepines. For example in the case of tetrazepam: all substances used increase largely the extrinsic Cotton effects of this drug whereas a displacement from same binding sites would cause a decrease of the extrinsic Cotton effects. On the assumption that five binding sites contribute to the Cotton effects, it seems very unlikely that no competition could be observed, because competition was observed with other ligands bound to more than one binding sites of the BSA molecule, e.g. methyl orange [24], trinitrobenzene-sulphonic acid [25], and 1-anilino-8-naphthalenesulfonate [26, 27]. These observations too support our suggestion that there are on the BSA molecule, less than five binding sites contributing to the extrinsic Cotton effects. Furthermore, the inversion in sign of the extrinsic Cotton effects (Table 2) cannot be explained by competition. The origin of these alterations of the extrinsic Cotton effects therefore must be that all these substances have a qualitatively similar influence on the protein conformation. In case of each of the substances the influence on the extrinsic Cotton effects of all benzodiazepines is similar. The intensity of this spectral changes provoked by the substances follows the range: caprylic acid < oleic acid < stearic acid < dodecyl sulfate. It is not clear if there are only small changes of the environment of the binding sites or if these alterations are an unfolding of the protein, as reported for some of the substances [28, 29]. There is evidence for an unfolding mechanism, because the influences of the fatty acids and dodecyl sulfate are very similar to the influences of pH on the CD spectra (Fig. 4), and because it is known that the origin of these changes, the N → B transition, is an unfolding mechanism [20].

In summary, whereas the gel filtration studies have shown very large differences of the binding of the benzodiazepines to bovine and human serum albumin, the CD studies suggest at least for a part of the benzodiazepine binding sites on BSA that there is one molecular mechanism of the binding and that the binding sites themselves have some properties in common. In regard to the discussion in the preceding paper [9], the origin of the large differences of both albumins must be more the existence of a greater number of binding sites on the BSA molecule than the failure of the specific binding site on the BSA molecule. The large differences in the interaction of HSA and BSA with the benzodiazepines found with the gel filtration technique [9] are of pharmacokinetic importance. But this method is of limited value to gaining insight in the molecular mechanism of the binding process. The results indicate that it is very important to use more than one method for drug binding studies to get careful information on the binding.

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